CRITICAL FLUID OPTIONS FOR THE EXTRACTION AND ENRICHMENT OF NUTRACEUTICALS

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ABSTRACT

The recent development and commercialization of nutraceutical and functional food ingredients from a variety of agricultural resources has provided a unique opportunity for the utilization of critical fluid technology. Aside from the environmental benefits of using critical fluid media as a process agent, there is also the appeal to the consuming public from the perspective of ingredient safety, for consuming products obtained using only carbon dioxide, water, or GRAS (generally regarded as safe) solvents. In this presentation, we report our latest research utilizing a variety of extraction, fractionation, or reaction options to produce products for commercial use. Thermal gradient fractionation columns using several different modes of operation have been used to enrich sterol ester components from a variety of seed oil sources, as well as to remove unwanted components, such as fatty acids, from the resultant extracts. Precise control of the variables effecting fractionation column performance were critical for obtaining the reported enrichments, since many of the nutritionally-beneficial ingredients are present in only small quantities in the starting seed-based materials.

Higher enrichment of the trace nutritional ingredients can be achieved by using the preparative mode of supercritical fluid chromatography (SFC) in conjunction with extraction. This concept was demonstrated for the combined SFE/SFC enrichment of sterol esters and phospholipids, using a form of gradient elution chromatography in which SC-CO_2 is the principle component of the mobile phase. Finally, the exploitation of reaction chemistry using critical fluid media was used to directly synthesize sterol or fatty acid esters, for incorporation into food matrices. This is conveniently accomplished by using enzymatic catalysis in order to preserve the natural and benign nature of the synthesis process and resultant product, which can be conveniently combined with downstream critical fluid extraction and fractionation.

INTRODUCTION

Nutraceuticals, as the name suggests, are ingested substances which combine the benefit of food nutritional requirements, while offering some aspect of therapeutic protection to the human body. Such foods and natural substances are called functional foods, designer foods, pharma foods, as well as many less elegant descriptors. Functional foods are similar in appearance to conventional foods, are consumed as part of a normal diet regime, and have demonstrated physiological benefit, i.e., reducing the risk of a disease state. Naturally-derived products are purchased to enhance stamina and energy, for weight control, to avoid illness, and to compensate for the lack of exercise. Depending upon the definition of a nutraceutical, the market ranges of such products is conservatively estimated to be 3.15 - 4.6 billion dollars in the USA and range from 1.05-1.6

billion US dollars in Europa. A broader definition of "functional" food pegs their US market value between 14.2-17.6 billion US dollars, and if one assumes that 50% of the food selected for consumption is based on health or medical considerations, then the estimated value of the nutraceutical market expands to 250 billion US dollars [1].

Consumers of nutraceuticals have expressed concern about pesticide or chemical residues, processing technology that contributes to ecological pollution, antibiotics or growth hormones in their foods, and the extensive use of preservatives in the foods they consume. It is for these reasons that technologies incorporating the use of critical fluids become important in the production of nutraceutical ingredients. Critical fluids, such as carbon dioxide (SC-CO_2), SC-CO_2/ethanol mixtures, and subcritical water, are environmentally benign processing agents; leaving no solvent residues in the final products, while minimizing the oxidation or degradation of thermally-labile components. Even a cursory inspection of the common classes of nutraceutical agents: herbs, speciality oils, plant extracts, specific protein fractions, and antioxidants, suggest a link between the two fields. Table 1 lists some of the common and popular nutraceutical agents in use today, their application, and the use or potential for using critical fluid processing in their production. Indeed, a segment of the production capacity of the 32 critical fluid processing worldwide is devoted to servicing the nutraceutical market.

Critical fluid processing can be used in several modes for producing nutraceutical ingredients or functional foods. Exhaustive extraction in which SC-CO_2 or a SC-CO_2-cosolvent mixture is used to yield an extract equivalent to those obtained with organic solvent extraction or pressing/expelling technologies [2] is well documented in the recent literature [3]. Fractional extraction where extraction pressure, temperature, time, or the addition of a cosolvent is varied on an incremental basis, is also capable of producing extracts that are somewhat either enriched or depleted in the desired nutraceutical agents [4]. Such fluid density-based or cosolvent-assisted extractions frequently yield extracts with considerable extraneous material; indeed specifically extracting or enriching a desired solute out of natural product matrix is somewhat akin "to finding a needle in a haystack". This problem is shown in Table 2 for naturally-occurring oils which have been extracted with SC-CO_2, however the desired "nutraceutical" components in the oil, or SC-CO_2 extract, are usually at very low concentration levels.

To enrich the concentration of desired component(s), researchers have resorted to fractionation techniques utilizing critical fluids. One of the simplest is separation of the extract with the aid of multiple separators held at different combinations of temperatures and pressures [5]. Using such an approach, the fractionation of essential oils from waxes and oleoresins has been accomplished. The use of fractionation columns in which a temperature gradient is imposed on a solute-laden flowing stream of SC-CO_2, either in a batch or countercurrent mode, is now being widely practiced. This methodology has been used for the production of fish oil concentrates [6], fractionation of peel oil components [7], and glyceride fractionation [8]. The coupling of critical fluids with chromatography on a preparative or production scale offers another alternative route to producing nutraceutical-enriched extracts. These chromatographic-based separations range from simple displacement or elution chromatographic schemes, i.e., the removal of cholesterol [9], to the more sophisticated simulated moving bed technology [10]; the latter technique perhaps is more favored for the purification of pharmacological compounds.

Table 1. Nutraceutical agents with respect to their use and processing with critical fluids.

Nutraceutical	Utility	Processed Via Critical Fluids
Saw Palmetto	Prostate	Yes
Kava-Kava	Anxiolytic	No
Hawthorne	Cardiotonic	No
Ginseng	Tonic	Yes
Garlic	Circulatory	Yes
Ginko Biloba	Cognitive	No
St. John's Wort	Depression	No
Chamomile	Dermatological	Yes
Echinacea	Colds/Flu	Yes
Black Cohosh	Gynecological	No
Lutein	Macular Degeneration	Yes
Flavanoids	Anti-Cancer	No
Isoflavones	PMS, Circulatory	No
Omega 3 EFA, DHA	Circulatory	Yes
Evening Primrose	Inflammation	Yes
Phytosterols	Circulatory	Yes
Tocopherols	Antioxidant	Yes
Phospholipids	Cognitive	Yes

Table 2. Natural oils extracted with critical fluids and their nutraceutical components.

Natural Oils	Nutraceutical Component
Rice bran	n-6, n-3 Fatty acids
Safflower	Phystosterols
Marine	Tocopherols
Sesame	Carotenoids
GLA-enriched	Phospholipids
Oat	Tocotrienols
Almond	Oryzanol
Wheat germ	Sesamolin
Amaranth	Glycolipids
Essential	Conjugated fatty acids
Avocado	Lipoproteins
Grapeseed	
Macademia nut	
Kiwi	
Genetically-modified oils	

Although there are many examples for processing nutraceutical ingredients using critical fluids, we will discuss here our research for producing extracts, enriched fractions, and products containing sterols and steryl esters from natural and synthetic sources. Such moieties have been clinically-evaluated [11] for their proven effectiveness in inhibiting cholesterol absorption and synthesis in the human body, and resulted in the marketing of two well known products, "Benecol" and "Take Control" worldwide[12]. The former product is obtained via a proprietary process involving the isolation of plant sterols from tall oil followed by synthetic modification, while the latter product is produced commercially from soybean oil feedstock. In addition, an alternative synthetic route for producing steryl esters via enzymatic catalysis in SC-CO_2 will be presented, thereby offering a "naturally" synthesized nutraceutical agent for incorporation into functional food compositions.

MATERIALS AND METHODS

An assortment of equipment and instrumentation on different scales were used in these related studies, hence only a generic description of the techniques and methodology employed can be provided in these proceedings. A more detailed description of individual techniques can be found in the cited references or by contacting one of the authors. Initial verification of the applicability of a particular processing method was often accomplished with the aid of small scale extraction equipment, originally designed for analytical purposes. Rapid assessment of the applicability of specific extraction, fractionation, or reaction conditions could be achieved by using such instrumentation in a combinatorial mode, which has been described in the literature [13].

Some of the vegetable oil feedstocks used in the column fractionation and supercritical fluid extraction/supercritical fluid chromatography (SFE/SFC) experiments were exhaustively extracted using the 12-Liter NCAUR semi-continuous pilot plant [14]. Soybean, rice bran, and several different types of corn oil were prepared by extraction with SC-CO_2 either at 34.5 MPa and 40°C or at 68 MPa and 80°C. Approximately 1.5 kg of comminuted seed or fibre/germ matrix was placed in each of 4-Liter vessels that comprise the pilot plant system. When required, derived extacts were centrifuged to separate the oil from the water or wax in the CO_2-derived extract.

Fractionation column experiments were done in the semi-batch mode using a 1.70m high x 1.43 cm i.d. column, filled with Propak stainless steel packing. The column was operated in the isobaric mode, usually between 10-34 MPa, both with and without an ascending temperature gradient (40 - 90°C). Carbon dioxide flow rate through the column was in the range of 1.2-2L/min, expanded gas flow. Liquid oil was injected into the column at the top of the first of four separately heated zones using a mini, air-driven booster pump. Details of the apparatus and technique are available in the literature [15,16]. In some cases, a two-step semi-continuous process was used to fractionate fatty acids from the rest of the oil mixture. This was accomplished by doing an initial fractionation at 13.6 MPa and temperature gradient of 40/60/70/80°C followed by fractionation of the fatty acid-depleted oil at 20.5 MPa using an identical temperature gradient on the column.

Initial extraction conditions for the SFE stage of an integrated two-step, SFE/SFC process were evaluated with the aid of an Isco Model SFX-3560 automated extractor. Using the microprocessor controller, a matrix of different extraction conditions could be evaluated in a short time with respect to temperature, pressure, the effect of cosolvent addition, and extraction

time. The substrate of interest (in this case corn fibre or bran) was loaded into 10cc extractor cells prior to initiating automatically, the various extractions. A typical range of extraction parameters that would be tested would include runs at 13.8, 34.5, and 69 MPa and temperatures of 40, 60, and 80°C. Similarly, the Isco 3560 unit was also used to evaluate the feasibility of fractionating deodorizer distillate components (from rice bran) over the range of 10-34 MPa and 40-80°C, with and without ethanol (EtOH) as a cosolvent. In this case, four fractions were collected at 15 min intervals

The evaluation of the conditions for the SFC step was initially done on the Isco Model SFX 3560 unit, in which the extraction cartridges (10 mL) were filled with the test sorbent from 3 -5 g. This permitted the rapid screening of elution conditions as well as various sorbents. One of the most versatile sorbents that we have found for fractionating steryl esters and sterols from other lipophilic components is an aminopropyl-bonded silica. An oil sample (either extracted by SC-CO_2 or neat) is placed at the top of the sorbent-filled cartridge and placed in the carousel of the automated extractor. A sequential elution program is setup with the aid of the instrument's microprocessor, by which the eluent composition is changed in a stepwise fashion for a specified period of time. Fractions are then collected every 60-90 min. at specific pressure and temperature conditions. An example of this sequential elution program is show in Table 3 for the supercritical fluid fractionation of corn fibre oil. Here the initial fraction is collected over a 60 min time interval using neat CO_2, followed by fractions collected under conditions where the addition of ethanol to the SC-CO_2 eluent has been increased up to 10 vol. %. Chromatography is performed at 34.5 MPa and 40°C for these stepwise intervals followed by fraction collection in the automated receiver vessel tray [17].

The lipase-catalyzed synthesis of steryl esters was accomplished using an Isco SFX 2-10 unit extractor, equipped with a 2.5 mL extraction vessel which was used as a flow through reaction chamber. Typically about 750 mg of supported enzyme were placed in the extraction vessel to conserve enzyme. Reactant chambers or reservoirs were placed in line with two syringe pumps feeding SC-CO_2 to the reaction chamber, into which sterol and fatty acid were placed, respectively. After solvation into the SC-CO_2, the reactants were transported to the extractor vessel where the esterification occurred to form the desired steryl ester. Esterifications were conducted over the range of 20.6-31 MPa and temperatures ranging from 40-60°C. Chirazyme L-1 lipase proved to the most efficient catalyst for steryl ester synthesis. A detailed schematic of the experimental apparatus is shown in Figure 1 [18].

Table 3. Chromatographic parameters for the supercritical fluid fractionation of corn fibre oil.

Fraction	Pressure(MPa)	Temperature (°C)	Time (min)	Solvent(vol%)
1	69.0	80	60	CO_2
2	34.5	40	60	1%EtOH/CO_2
3	34.5	40	60	2%EtOH/CO_2
4	34.5	40	90	10%EtOH/CO_2
5	34.5	40	90	10%EtOH/CO_2

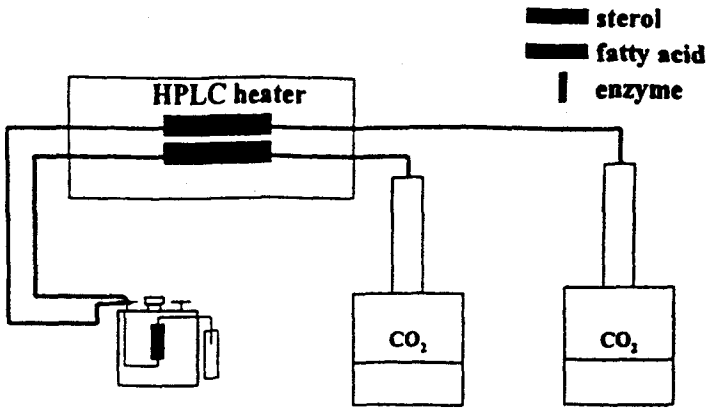

Figure 1. Continuous reaction system for the synthesis of sterol/stanol esters in SC-CO_2.

RESULTS AND DISCUSSION

Nutraceutical-based products or ingredients can be achieved using critical fluid technology in several modes. As noted previously, extracts or foods fortified with steryl esters or sterols are for their cholesterol-lowering properties. Three techniques have been under investigation in our laboratories to achieve the above goal: column fractionation, coupling selective SFE with SFC, and direct synthesis of steryl esters in SC-CO_2. In addition, removal of compounds such as fatty acids can be accomplished using one or more of the described techniques. However fractionation is not always necessary, since in some nutraceutical products (e.g., saw palmetto), there appears to be a synergistic effect of extracts containing both fatty acids and steryl esters.

For example, crude rice bran oil can be fractionated to remove unwanted fatty acids without a significant loss of oryzanol or triglyceride content using a semi-batch fractionating column mode. Extracts with the highest free fatty content (36.6 %) were achieved at 20.5 MPa and 80°C by operating the fractionating column in an isobaric mode. Raffinate samples showed increased amounts of triglycerides and sterols, and reduced levels of free fatty acids (2.9 - 5.1 %). Column fractionations conducted over an extended time period further resolved the free fatty acid moieties from the triglycerides, yielding extracts after 8 hours of operation of almost 60 % free fatty acid content. This is 8-fold enrichment relative to the free fatty acid content of the starting rice bran oil which was 7 % free fatty acids. Using this technique, it was possible to produce a raffinate fraction containing 95 % triglycerides, <1 % free fatty acids, 0.35 % free sterol, and 1.8 % oryzanol content, at 13.6 MPa and 45°C. This composition compares favorably with the content of commercially-refined rice bran oil or high oryzanol rice bran oil, and in addition contains three times as much oryzanol.

A more striking result can be achieved by superimposing a thermal gradient on the fractionating column. For example, the free fatty acid content of crude rice bran oil can be reduced from 7 to 0.5% at 13.6 MPa using a four-zone thermal gradient of 40/60/70/80°C. The raffinate of this initial fractionation step can then be further fractionated at 20.5 MPa using the above thermal gradient to yield a product whose steryl ester content exceeds that found in a commercially-available, steryl ester-enriched margarine. This two step fractionation process is illustrated in Figure 2. Similar results have been achieved using corn fiber oil as a starting substrate in which the phytosterol content is increased from 6 to 19% using the above two-step enrichment process. Again, the result is a lipophilic-based composition that is similar to a commercially-available phytosterol-enriched margarine having a low free fatty acid content (<0.5%).

Side streams from conventional oilseed processing also present a rich source of nutraceutical ingredients. Processes such as degumming, deacidification, bleaching, and deodorization remove valuable nutritional ingredients from the final cooking or salad oil. Estimates of their removal are as follows [19]: phospholipids (>95%), steryl esters (>95%), free fatty acids (>95%), pigments, i.e., carotenoids (>90%), sterols (32-61%), tocopherols and tocotrienols (35-47%). For this reason, we and others have applied extraction [20], fractionation [21], and reaction [22] techniques using supercritical fluids to distillates and sludges containing the above compounds. Figure 3 shows the effect of the column fractionating pressure on the composition of rice bran deodorizer distillate (DD), with the wax fraction removed, at 45°C. As noted in Figure 3, selection of fractionation pressure allows one to obtain an extract of specific composition in terms of free fatty acid (FFA), phytosterol (St), and triglyceride (TG) content. Therefore, depending on the pressure selected (13.6 MPa vs. 27.2 MPa), one could reduce the FFA content by 20%, double the phytosterol content, and increase the TG content eight-fold in the resultant extract. This ability to custom design extracts using a columnar supercritical fluid fractionation technique is particularly attractive in achieving an optimal therapeutic composition in a nutraceutical extract. A two-step enrichment process (13.6 MPa/45°C followed by a 34.0 MPa/80°C step) can produce extracts with > 30 % phytosterols and reduced free fatty acid content from rice bran or soybean oil deodorizer distillates

Coupling SFE with SFC is a powerful technique for the enrichment of extracts containing nutraceutical ingredients. Previously we have shown that tocopherols can be enriched a factor of 1.8 - 4.3 over their concentration in soybean oil using optimized conditions for the SFE step [23]. Further enrichment is achieved by transporting the SFE-enriched extract to the SFC stage, where enrichment factors relative to the individual tocopherol content in soybean oil are 2.4-30.8, depending on the individual tocopherol. Similarly, phospholipid concentrates can be prepared via SFE/SFC, resulting in individual fractions containing 75% of a specific individual phospholipid [24]. Using a similar approach, SFE/SFC was used to enrich steryl esters from corn bran and fiber oils.

In the case of the corn bran oil, it was determined combinatorially with the aid of the automated analytical SFE instrument, that two sets of conditions: 69 MPa/80°C and 34.5 MPa/40°C were optimal for enriching the steryl esters. The resultant extracts contained 1.25 % ferrulate phytosterol esters. Using an aminopropyl silica-based sorbent, the initial extract from the corn bran oil was fractionated using a similar program to that described in Table 3. Initially the SFE-derived extract was chromatographed at 69 MPa and 80°C to remove most of the interfering triglycerides. Then the pressure and temperature were lowered to 34.5 MPa and 40°C, respectively, and ethanol cosolvent added stepwise to achieve fractions enriched in phytosterol

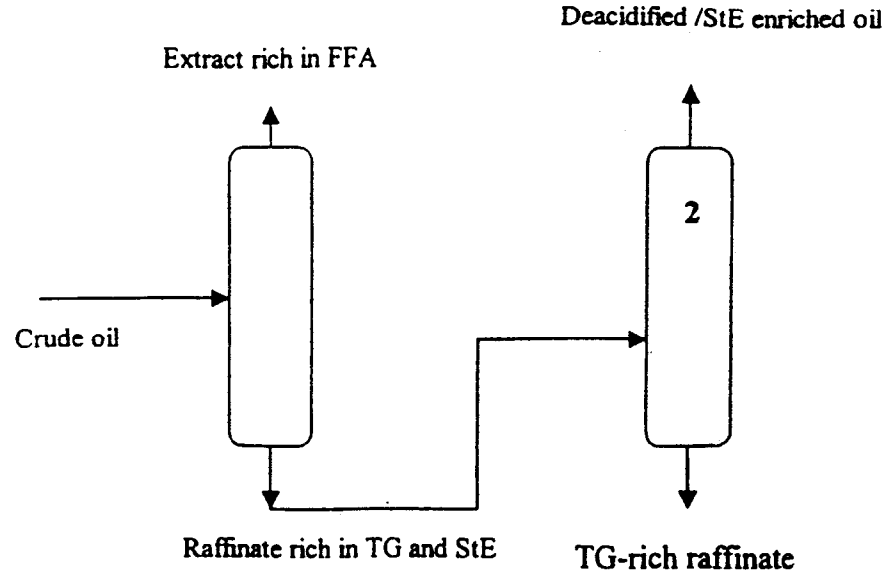

Figure 2. Schematic diagram of a two-step columnar fractionation process for steryl ester enrichment in vegetable oils.

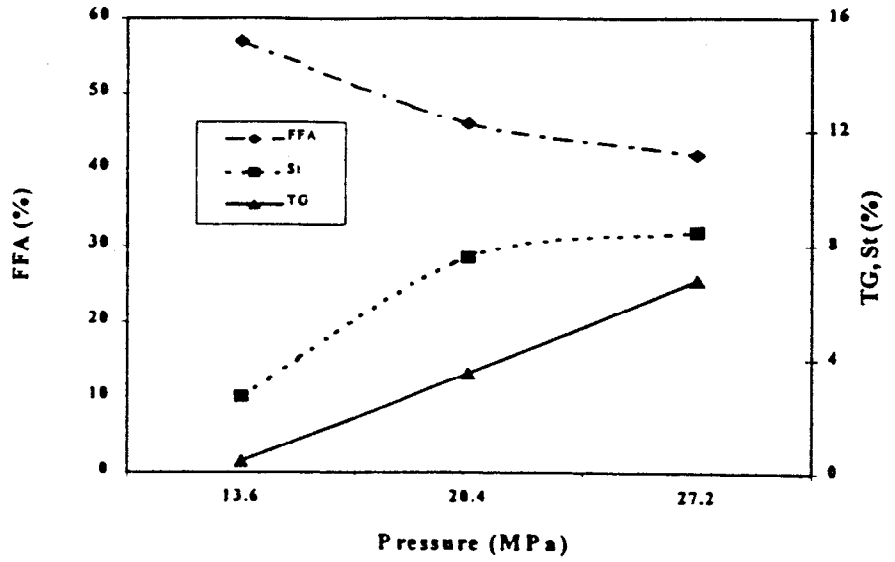

Figure 3. Effect of temperature on the composition of the extract fraction collected during the supercritical fluid fractionation of rice bran deodorizer distillate at 45°C.

content. Using this approach, ferulate phytosterol esters could be enriched to a 14.5 wt. % level.

As in the case of the deodorizer distillates, starting with a substrate containing a higher level of the nutraceutical targeted for enrichment can be advantageous. For this reason, the SFE/SFC process was applied to corn fiber oil, which has been reported to contain up to four times as much ferulate phytosterol esters as the previously-mentioned corn bran oil. However corn fiber itself contains only a few percent oil; in fact SFE at 34.5 MPa and 40°C yielded only 0.56 wt. % based on the weight of the corn fiber. Using the stepwise gradient program given in Table 1, it was found that the first fraction collected contained 15 wt. % fatty acid sterol esters and 85 wt. % triglycerides. Further elution of the starting substrate resulted in a final fraction consisting mostly of ferulate phytosterol esters, free fatty acids and sterols, and minor amounts of diglycerides.The weight percent of ferulate phytosterol esters in this fraction was 53%, twice the amount found in the starting corn fiber oil. Such an enriched concentrate has the potential to be used as an additive in a functional food formulation. It should be noted that for both the corn bran as well as corn fiber SFE/SFC studies, that the average mass balance for all of the corn fiber or bran components was close to 100 % recovery. This is an important factor when using SFC in the preparative or production mode.

An alternative route for producing nutraceutical ingredients is via synthesis. Here again the versatility of critical fluid technology can be exploited to produce the desired moiety using only natural materials. As described in the Materials and Methods section, a modified analytical SFE extractor was used to optimize the reaction conditions, as well as to save on time and expensive reagents, such as the lipase and sitostanol. Four enzyme candidates were initially screened using a batch, semi-continuous method [18]: Chirazyme L-1, Chirazyme L-3, Lypozyme IM, and Novozyme SP 435. Using the esterification between palmitic acid and cholesterol as a model reaction, it was ascertained that Chirazyme L-1 was the optimal lipase for the desired reaction. Using the same reaction, a series of reaction pressures and temperatures were surveyed in order to establish the optimal parameters for synthesizing the desired sterol esters. Using the batch, semi-continuous method with an initial static hold time of 5 min., it was determined that a pressure of 27.6 MPa and 50°C were optimal conditions for the production of the target sterol esters. Static hold time and flow rate were also studied with respect to ester yield and 5 min and a flow rate of 1.0 mL/min (multiply by approx.. 500 to get expanded CO_2 flow rate) were found to be optimal.

Using the above conditions, a series of reactions were run using cholesterol and sitostanol as sterol substrates with fatty acids ranging in chain length from C_8 to C_{18}. In all cases yields in excess of 90% were attained, and greater than 98% yields for the C_{10}-C_{18} esters of cholesterol and C_{16}-C_{18} esters of sitostanol, respectively. The latter result is particularly significant since it is a sitostanol ester that has been reported to be the active cholesterol-lowering ingredient in a commercially-available margarine spread [25]. Similar results were also obtained when using the system depicted in Figure 1 in the continuous flow mode. Again, yields in excess of 80% were achieved in all cases after only 15-20 minutes of run time. This bodes well for the synthetic production of nutraceutically-significant sterol esters using a "green" approach consisting only of SC-CO_2 and a natural enzyme catalyst.

CONCLUSIONS

The above methodology demonstrates the effectiveness of critical fluid technology for producing nutraceutical-containing products or ingredients. Although SFE alone has been

successful in producing some of these products [26], it is anticipated that fractionation and enrichment techniques will play an increasingly important role in "customizing" extracts or fractions for use in the functional food field. Columnar fractionation techniques are very effective for producing a nutraceutical-containing extract of a defined and perhaps synergistic composition. It is important to realize that for many applications in the nutraceutical field, that ultra-high purity extracts are usually not required, but attaining a significant enrichment of the nutraceutically- active ingredient over that present in its initial composition provides additional flexibility to the food formulator.

All the described techniques in this contribution are attractive "green" processing options whether one is considering column fractionation, SFE/SFC, or the natural synthesis of sterol esters. This coupled with the elimination of any harmful chemical residues and the prophylactic action of CO_2 on the final product, suggests a bright application future for critical fluid technologies in the nutraceutical field.

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SOURCE: Proceedings of the 7th Meeting on Supercritical Fluids: Particules Design, Materials and Natural Products Processing. Tome 2: Natural Products and Others. December 6,7,8, 2000, Antibes/Juan-Les-Pins, France. International Society for the Advancement of Supercritical Fluids / ISASF. Chairmen: M. Perrut and E. Reverchon (Institut National Polytechnique de Lorraine)
ISBN 29052673310 p. 537-547. 2000